Chlorophyll extraction from leaves, needles and microalgae: A kinetic approach

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Abstract: Currently, there is a strong focus on industrial production of chlorophyll as a natural pigment. Two factors are required in the economically feasible process to make chlorophyll production: material with high pigment content and efficient extraction mechanism. In this work, extraction of chlorophyll from harvested black locust (*Robinia pseudoacacia*) leaves, Scots pine (*Pinus sylvestris*) needles, field sow thistle (*Sonchus arvensis*) leaves, and green microalga (*Chlorella* sp.) was discussed. The highest pigment content was detected in *Chlorella* cells (4.46%) followed by black locust leaves (1.63%), sow thistle leaves (1.48%) and pine needles (0.38%). The chlorophyll extraction rate was the highest for black locust leaves (k = 3.59 h^{-1}), sow thistle leaves (k = 2.90 h^{-1}) and *Chlorella* cells (k = 2.80 h^{-1}) with the use of methanol as a solvent. In investigated materials, needles showed higher resistance for chlorophyll extraction (k = 0.93 h^{-1}) when compared to leaves and microalgae. Values of extraction kinetic constant were much lower for all materials (0.22 – 1.12 h^{-1}) in the case of using ethanol as a solvent. Black locust leaves and *Chlorella* cells were proved to be the most attractive materials for chlorophyll production.

Keywords: chlorophyll, extraction, leaves, needles, microalgae, kinetic approach

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1 Introduction

Plant materials are a vast source of natural compounds that can be harnessed for human benefits. Leaves, needles and microalgae contain pigment components such as chlorophylls and carotenoids. These pigments showed applications in food, pharmaceutical or cosmetic industry[1-3].

Chlorophyll is the most common pigment found in higher plants and algae. In chlorophyll structure, four pyrrole rings are combined to form a scaffold with a central magnesium ion and a long phytol chain. Two types of chlorophyll, *a* and *b*, are present in terrestrial plants and green microalgae. The difference between these two types of molecules stems from a single side group attached to basic chlorophyll structure. Chlorophyll *a* contains a methyl group; chlorophyll *b* contains a formyl moiety. Chlorophyll absorbs light in the red and the blue-violet regions of solar spectrum. Green light is not absorbed but reflected giving chlorophyll its green color. In plants, chlorophyll molecules harvest light in the process of photosynthesis that converts light energy into chemical energy[4]. Carotenoids are pigments that belong to the category of tetraterpenoids. In plants, carotenoids are components of the photosynthetic apparatus, where they absorb blue-green light and increase the range of sun light used for photosynthesis. Additionally, carotenoids serve as a protection for photosynthetic mechanism against oxidative stress and photodamage. There are over 600

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carotenoids known to be present in nature[5].

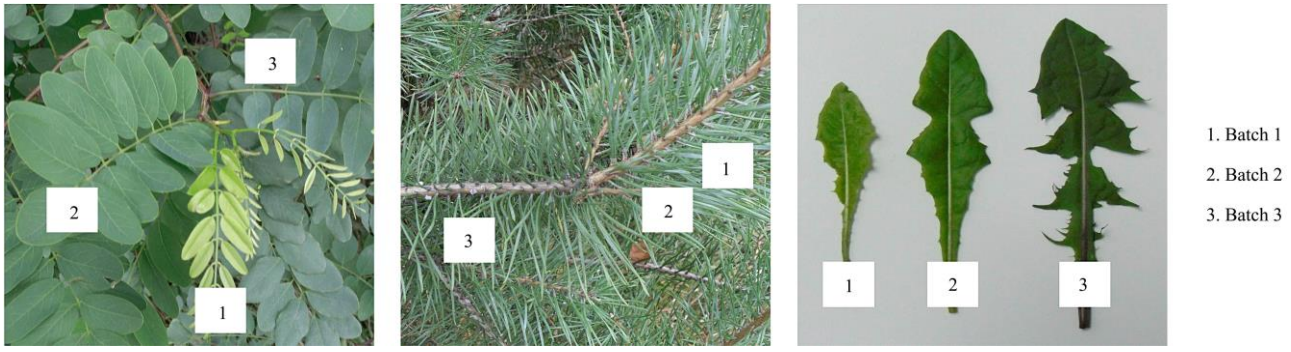
Black locust (*Robinia pseudoacacia*) is a fast growing deciduous tree that belongs to a family of *Fabaceae*. Leaves of *Robinia* are blue-green, alternately arranged, in odd-pinnate elliptic type with slender hairy petioles[6]. Scots pine (*Pinus sylvestris*) from *Pinaceae* family, is an evergreen coniferous tree spread in many climatic zones. Pine needles, produced in fascicles of two, are light or dull green, thin and rigid in structure, smooth on the surface, sharp on the top[7]. Field sow thistle (*Sonchus arvensis*), a member of *Asteraceae* family, is a deep rooted perennial weed commonly found in cultivated fields, pastures, woodlands, roadsides, and lawns. *Sonchus arvensis* leaves develop from a basal rosette and vary in size, shape and green hue. Leaves are alternate, have pointed lobs with prickly edges, clasp the stem[8]. *Chlorella* strains, eukaryotic, unicellular, green microalgae, are representatives of aquatic plankton[9]. Plant materials mentioned above are common inhabitants of terrestrial or aquatic environment and therefore can be easily harnessed for chlorophyll production.

Recently, microalgae are the object of intensive investigations in the aspects of biofuels[10,11], because they represent the valuable source of bioactive compounds, among others chlorophyll. Different pigment extraction techniques including organic solvent extraction, as well as supercritical fluid extraction, were tested for various microalgae strains[12]. However, no research has compared so far the chlorophyll extraction from microalgae and leaves or needles. Therefore, this paper is aimed at comparison of different sources of chlorophyll and their extraction rate from various natural materials. A kinetic model of chlorophyll extraction from leaves, needles and microalgae is depicted and extraction process according to different materials and solvent used is described. This method enables proper selection of material and solvent to produce high amount of chlorophyll in the shortest possible time. Microalgae seem to be the most promising alternative source for chlorophyll.

2 Materials and methods

2.1 Leaves and needles

Black locust (*Robinia pseudoacacia*) leaves, Scots pine (*Pinus sylvestris*) needles and field sow thistle (*Sonchus arvensis*) leaves were harvested in a central region of Poland (51°51' N, 19°25' E). The age of harvested leaves and needles varied from young to mature representatives. In order to distinguish leaves and needles of different age, plant materials were collected in separate batches. Each material type possessed three independent batches (Figure 1). This evaluation was made according to the size and color intensity of every harvested plant samples. Black locust and sow thistle leaves were current year materials (all batches). Pine needles were current year (Batches 1 and 2) and previous year materials (Batch 3). Material batches were immediately transported to laboratory, cut with scissors into small pieces (< 5 mm^2) and used for water content determination and extraction process.



a. Black locust leaves b. Scots pine needles c. Sow thistle leaves

Figure 1 Different batches of plant materials

2.2 Microalgae

Chlorella sp. was obtained from Culture Collection of Baltic Algae (CCBA). The stain was cultivated in a modified Bold's Basal Medium (BBM). Medium

composition was as follows: K_2HPO_4 (0.075 g/L), KH_2PO_4 (0.175 g/L), $MgSO_4 \cdot 7H_2O$ (0.075 g/L), $NaNO_3$ (0.25 g/L), $CaCl_2 \cdot 2H_2O$ (0.025 g/L), NaCl (0.025 g/L), $Na_2EDTA \cdot 2H_2O$ (0.025 g/L), $FeCl_3$ (6 mg/L), H_3BO_3 (1 mg/L), $ZnSO_4 \cdot 7H_2O$ (1.4 mg/L), $MnSO_4 \cdot H_2O$ (0.16 mg/L), $CuSO_4 \cdot 5H_2O$ (0.16 mg/L), $CoCl_2 \cdot 6H_2O$ (0.065 mg/L), $Na_2MoO_4.2H_2O$ (0.135 mg/L). *Chlorella* (Figure 2) was cultivated in 500 mL round flasks with an initial culture volume of 300 mL, in an incubator (Certomat® BS-T) equipped with a light source (fluorescents lamps, 5 × 18 W) and a shaker (110 r/min). The culture was incubated in day/night (16 h/8 h) cycles during five weeks. Optical density (OD 530) and chlorophyll (*a* + *b*) concentration were measured in two independent cultures to follow *Chlorella* growth.

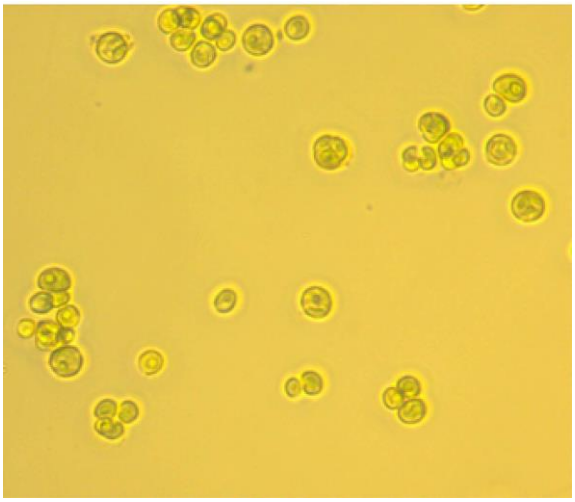


Figure 2 *Chlorella* sp. from Culture Collection of Baltic Algae (CCBA)

2.3 Water content measurement

Samples of leaves and needles were dried at 105°C in an oven for at least two hours for dry weight determination[13]. This measurement was repeated for at least two independent samples from each batch. Water content as percent of fresh sample material was expressed as:

$$W_{cont} = ((F_w - D_w)/F_w) \times 100\%$$

where W_{cont} is water content, %; F_w is fresh weight, g; D_w is dry weight, g.

2.4 Dry weight measurement

Samples of *Chlorella* culture were centrifuged (5 000 r/min, 5 min) to remove cultivation media containing salts. Pellets were submerged in distilled water, shaken and centrifuged to remove remnants of cultivation media. This step was repeated in triplicate. Purified samples were dried in the same way as for leaves and needles. Dry weight was measured and used to determine chlorophyll and total carotenoid content in *Chlorella* culture. This procedure was carried out for three independent samples.

2.5 Pigment extraction from leaves and needles

Equations for pigment determination with the use of methanol are given as follows:

$$Chl_a = 16.72 \times A_{665} - 9.16 \times A_{652} \quad (1)$$

$$Chl_b = 34.09 \times A_{652} - 15.28 \times A_{665} \quad (2)$$

$$Car_{total} = (1000 \times A_{470} - 1.63 \times Chl_a - 104.96 \times Chl_b)/221 \quad (3)$$

Equations for pigment determination with the use of ethanol are as follows:

$$Chl_a = 13.36 \times A_{664} - 5.19 \times A_{648} \quad (4)$$

$$Chl_b = 27.43 \times A_{648} - 8.12 \times A_{664} \quad (5)$$

Two solvents, methanol and ethanol, were applied to extract pigments from tested plant materials. Pigment amount in obtained extracts was measured spectrophotometrically (T80+ UV/VIS Spectrometer PG Instruments Ltd) for chlorophyll *a*, chlorophyll *b* and total carotenoid amount (Equations (1)-(3)), in case of methanol as a solvent and chlorophyll *a* and *b* (Equations (4) and (5)) when ethanol used[14]. Firstly, fresh material batches were extracted only with pure methanol (POCh) in sealed tubes kept at room temperature (23 ±1)°C in dark. Extraction was carried out for a period that enabled the complete removal of green color from treated materials. Results obtained from equations were compared with the amount of plant materials used for extraction and pigment content in tested batches was determined. Chlorophyll and total carotenoid content was expressed on dry material basis. These measurements were repeated for at least two independent samples from each batch. Freshly cut samples of black locust leaves, pine needles and sow thistle leaves from Batch 3 were also used in a kinetic model of chlorophyll extraction with two solvents as a comparison. Only samples from Batch 3 were tested in terms of chlorophyll extraction kinetics, because of the largest chlorophyll content. Moreover, lower pigment content in samples

from Batches 1 and 2 caused the difficulty to obtain reliable experimental data for kinetic model. Plant samples were carefully weighed in the equal amount of 250 mg. Further, 5 ml of pure methanol (POCh) or 95% V/V ethanol (POCh) was added to weighed materials. Experiments were performed in a set of laboratory tubes. During extraction process, plant material samples were stored at the bottom of laboratory tubes and chlorophyll molecules were freely released from materials. One laboratory tube was used only for one measurement of chlorophyll (a + b) dissolved in methanol (Equations (1) and (2)) or ethanol (Equations (4) and (5)). Before pigment determination, a proper laboratory tube was mixed for a moment in vortex to ensure equal chlorophyll concentration in the whole volume of solvent used. No centrifugation was necessary as plant materials were settled immediately after mixing. Tubes were taken at time intervals, simultaneously for methanol and ethanol with the same plant material. Experiments were performed at least in duplicate for each material and solvent used.

2.6 Pigment extraction from microalgae

Chlorella was cultivated in a batch culture to achieve higher amount of biomass and chlorophyll. After 32-day cultivation, 2.5 mL samples from culture was taken and added to laboratory tubes and centrifuged to remove media. After removing media, 5 mL of pure methanol or 95% V/V ethanol was added to tubes. Sealed tubes were shaken to ensure that microalgae cells are in the whole solvent volume. Conditions of chlorophyll extraction and measurement were the same as for leaves and needles with the exception of centrifugation. The process of centrifugation (5 000 r/min, 5 min) was necessary to separate cells from solvent, because floating cells caused interferences during chlorophyll measurement. The centrifugation time was included into chlorophyll release time, whilst determining kinetics of chlorophyll extraction from *Chlorella*.

2.7 Kinetic model

The kinetic model of chlorophyll (a + b) release during extraction process was depicted as a function of chlorophyll (a + b) released ft in time t ($ft = C_t/C_{max}$). Experimental points were approximated with first order kinetic model:

$$C_t = C_{max} \times (1 - e^{-kt})$$

where, C_t is amount of chlorophyll (a + b) released in t time, μg; C_{max} is maximum amount of chlorophyll (a + b) released during extraction process, μg; k is first order kinetic constant, h^{-1}.

2.8 Fitting of data and modelling

Microsoft Excel was used to compute linear regression, standard deviation and coefficient of determination in obtained experimental data.

3 Results and discussion

3.1 Characteristics of chlorophyll profile in different batches of leaves and needles

Experimental results showed considerable differences in the chlorophyll and carotenoid content among tested batches of plant materials (Table 1). The pigment level increased from Batch 1 up to Batch 3, with increasing the age of all plant materials. The biggest range of chlorophyll content between tested batches was attributed to black locust leaves and the smallest to field sow thistle leaves. However, the widest range of carotenoid content was reported for pine needles and the narrowest for black locust leaves. The highest overall chlorophyll content among tested materials was found for black locust leaves from Batch 3 (1.63%). Overall total carotenoid content was observed to be the highest both for black locust and field sow thistle leaves in Batch 3 (0.25%). The smallest chlorophyll concentration was detected for pine needles, where maximal chlorophyll content for Batch 3 (0.38%) was smaller than minimal content of this pigment (Batch 1) for black locust (0.52%) or field sow thistle (0.94%) leaves.

Chlorophyll a to b ratio values were higher for black locust leaves and pine needles than for field sow thistle leaves. It was reported that plants exposed to high irradiance contain chlorophyll a to b ratio above 3.0 and plants grown under diminished light intensity possess this ratio below 3.0[15]. Values of chlorophylls to total carotenoids ratio were the lowest for young material samples (Batch 1) and increased with the age of all investigated plant materials (Batches 2 and 3). The higher carotenoid content, in relation to chlorophyll

content for young materials in tested batches, may be explained by the fact that higher level of carotenoids protects susceptible young leaves and needles against excessive sun rays.

Table 1 Pigment and water content in black locust leaves, scots pine needles and sow thistle leaves

Black locust leaves *			
Parameters	Batch 1	Batch 2	Batch 3 **
Chlorophyll (*a* + *b*) (%)	0.52±0.020	0.965±0.045	1.625±0.005
Carotenoids (%)	0.145±0.005	0.215±0.005	0.245±0.005
Chlorophyll *a*/*b* ratio (-)	3.12±0.002	3.98±0.410	3.77±0.110
Chl (*a* + *b*)/Carot ratio (%)	3.54±0.045	4.52±0.150	6.62±0.120
Water content (%)	67.3±0.200	56.9±1.200	42.8±4.000
Scots pine needles *			
Parameters	Batch 1	Batch 2	Batch 3**
Chlorophyll (*a* + *b*) (%)	0.142±0.022	0.208±0.030	0.379±0.003
Carotenoids (%)	0.028±0.002	0.037±0.001	0.065±0.002
Chlorophyll *a*/*b* ratio (-)	3.214±0.011	3.395±0.300	3.305±0.090
Chl (*a* + *b*)/Carot ratio (%)	5.18±0.040	5.64±0.250	5.81±0.160
Water content (%)	47.5±1.300	49.3±3.300	39.8±2.100
Sow thistle leaves *			
Parameters	Batch 1	Batch 2	Batch 3**
Chlorophyll (*a* + *b*) (%)	0.940±0.030	1.170±0.010	1.475±0.015
Carotenoids (%)	0.190±0.010	0.225±0.005	0.245±0.005
Chlorophyll *a*/*b* ratio (-)	2.967±0.041	2.983±0.050	2.645±0.090
Chl (*a* + *b*)/Carot ratio (%)	4.98±0.073	5.1±0.060	6.09±0.135
Water content (%)	71.4±0.400	77.4±1.000	78.2±0.900

Note: * Pigment extraction was performed only with methanol;
** Batch 3 was further used to determine a kinetic model of chlorophyll extraction with methanol and ethanol.

Not only pigments, but also water content varied among investigated materials. The amount of water in tested materials was the highest for field sow thistles (71.4%-78.2%) and the lowest for pine needles (39.8%-47.5%). These two materials were found to exhibit stable water content regardless of batch controlled. On the other hand, black locust leaves showed the highest water content values for young samples in Batch 1 (67.2%) and the decrease in water contents with the increase of sample ages, which were 56.9% and 42.8% for Batches 2 and 3, respectively.

3.2 Characteristics of chlorophyll profile in culture of microalgae

Chlorella strain was cultivated for 32 days, during which the increase in optical density and pigment concentration in the culture was observed (Figure 3). During the first 24 days of cultivation, the increase in optical density was 0.04 per day, chlorophyll (*a* + *b*) and total carotenoid concentration were respectively 0.660 μg and 0.143 μg per day in every ml of culture. Between a 24 and 32 day of cultivation, there was the increase in optical density equal to 0.061 per day. Pigment concentration increased daily, 1.368 μg for chlorophyll and 0.294 μg for carotenoids between 24 and 28 cultivation days. Within the last four days, pigment concentration increased by 0.268 μg and 0.095 μg every day, reaching its maximal values 23.356 μg chlorophyll/mL culture and 5.322 μg carotenoids/ml culture. After 32 days, chlorophyll concentration shortly remained at the same level and started decreasing (data not shown). Values of chlorophyll *a* to *b* ratio and chlorophyll to carotenoid ratio varied from 2.77 to 3.94 and from 4.165 to 4.942, respectively, during *Chlorella* cultivation. The content of chlorophyll (*a* + *b*) and total carotenoids in dry biomass at a 32-day cultivation was measured to be respectively, around 4.46% and 0.97% (Table 2).

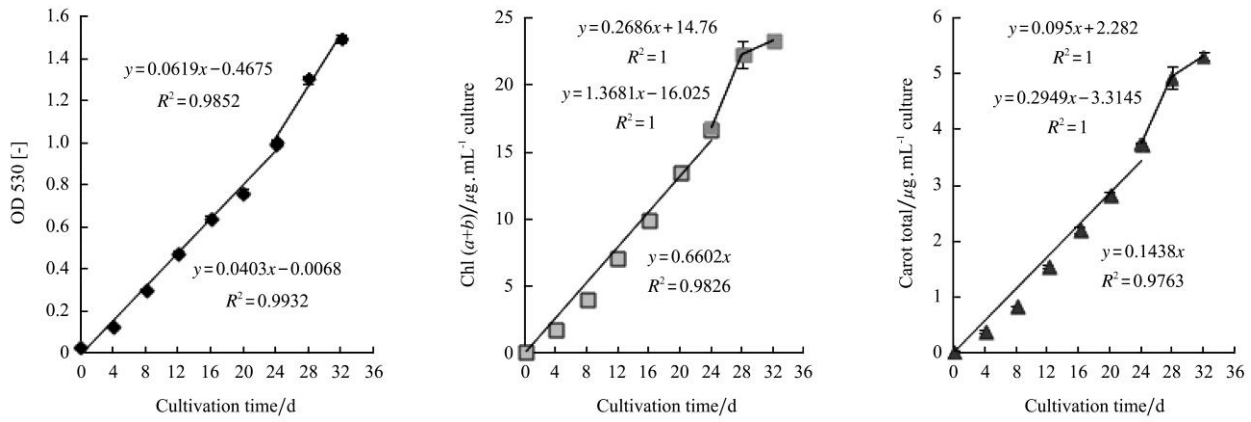


Figure 3 Cultivation of *Chlorella* sp. in a batch culture

Table 2 Pigment profile in *Chlorella* sp. culture after 32 days of cultivation

Chlorella sp. culture *	
Parameters	32-day cultivation **
Chlorophyll (*a* + *b*) (%)	4.460±0.057
Carotenoids (%)	0.970±0.011
Chlorophyll *a*/*b* ratio (-)	2.878±0.055
Chl (*a* + *b*)/Carot ratio (%)	4.613±0.031
Dry weight (mg/ml culture)	0.519±0.019

Note: * Pigment extraction was performed only with methanol;
** 32-day culture was further used to determine a kinetic model of chlorophyll extraction with methanol and ethanol.

3.3 Effect of plant material and solvent type on chlorophyll extraction rate

In plants, chlorophylls are arranged in a form of chlorophyll-proteins complexes. These complexes are localized in chloroplasts surrounded by protein-lipid bilayers[16]. The process of chlorophyll extraction from plant materials involves the use of organic solvents that diffuse through plant tissue, increase permeability of chloroplast membranes and cleave linkages between chlorophyll and protein molecules[17]. Results of our study showed that both methanol and ethanol at room temperature completely extracted chlorophyll from fresh tested materials. However, the time necessary to remove chlorophyll was different for each solvent used. The rate of chlorophyll release was expressed in a form of first order kinetic constant (k). The time, necessary to completely remove chlorophyll from a treated material, determined the value of kinetic constant. For kinetic study of chlorophyll release, leaves and needles from Batch 3 and a 32-day *Chlorella* culture were used, due to the highest pigment content and thus, the biggest potential for abundant chlorophyll production. From our observations, extraction process for Batch 1 was completed faster than for Batch 3 (data not shown), but because pigment content was much smaller, only Batch 3 was further studied.

In our study, chlorophyll extraction with methanol was carried out 2.5-4.3 times (leaves and needles, Figure 4) up to 10 times faster (microalgae, Figure 5) than with ethanol. According to literature data, ethanol molecules penetrate lipid-protein bilayers more efficiently than methanol due to higher hydrophobicity[18]. Based on those results, ethanol should be considered as a better solvent for chlorophyll extraction than methanol. However, results from our study are opposite. Hence, not ability for dissolving membranes, but another mechanism for chlorophyll release should be taken into consideration.

The time of chlorophyll release showed to be not only solvent, but also plant material dependent. The complete removal of chlorophyll (Figure 4) from black locust leaves with the use of methanol was slightly faster (k = 3.59 h^{-1}) than from sow thistle leaves (k = 2.90 h^{-1}) for the same solvent. It was suggested that high water content could result in diluting solvent and reducing efficiency of extraction process[19]. Higher water content in sow thistle leaves may explain this difference. However, during chlorophyll extraction with ethanol, results for these two types of leaves were opposite, with k = 1.12 h^{-1} (*Sonchus* leaves) and k = 0.83 h^{-1} (*Robinia* leaves). Moreover, chlorophyll extraction with both solvents was completed a few times faster for leaves when compared with needles, where the water content was smaller. It indicates that water content is not a crucial factor affecting extraction rate. In literature, the difference in leaf anatomy is mentioned to affect chlorophyll extraction from leaves and needles[20]. Black locust and field sow thistle belong to angiosperm dicot plants. The characteristics of dicot leaves are mesophyll structure between upper and lower epidermis. In dicot leaves, mesophyll is composed of palisade and spongy cells containing chloroplasts. Palisade mesophyll cells are densely arranged in structure. Structural organization of spongy mesophyll cells contains considerable intracellular space[21]. Scots pine is an example of gymnosperm plants. In pine needles, uniform mesophyll structure containing chloroplasts is surrounded by thick epidermal cell wall. Moreover, there is a hypodermis layer, composed of sclerified cells and localized below epidermis[22]. This stiffness of needle structure can create an additional barrier for organic solvent and resistance against chlorophyll extraction.

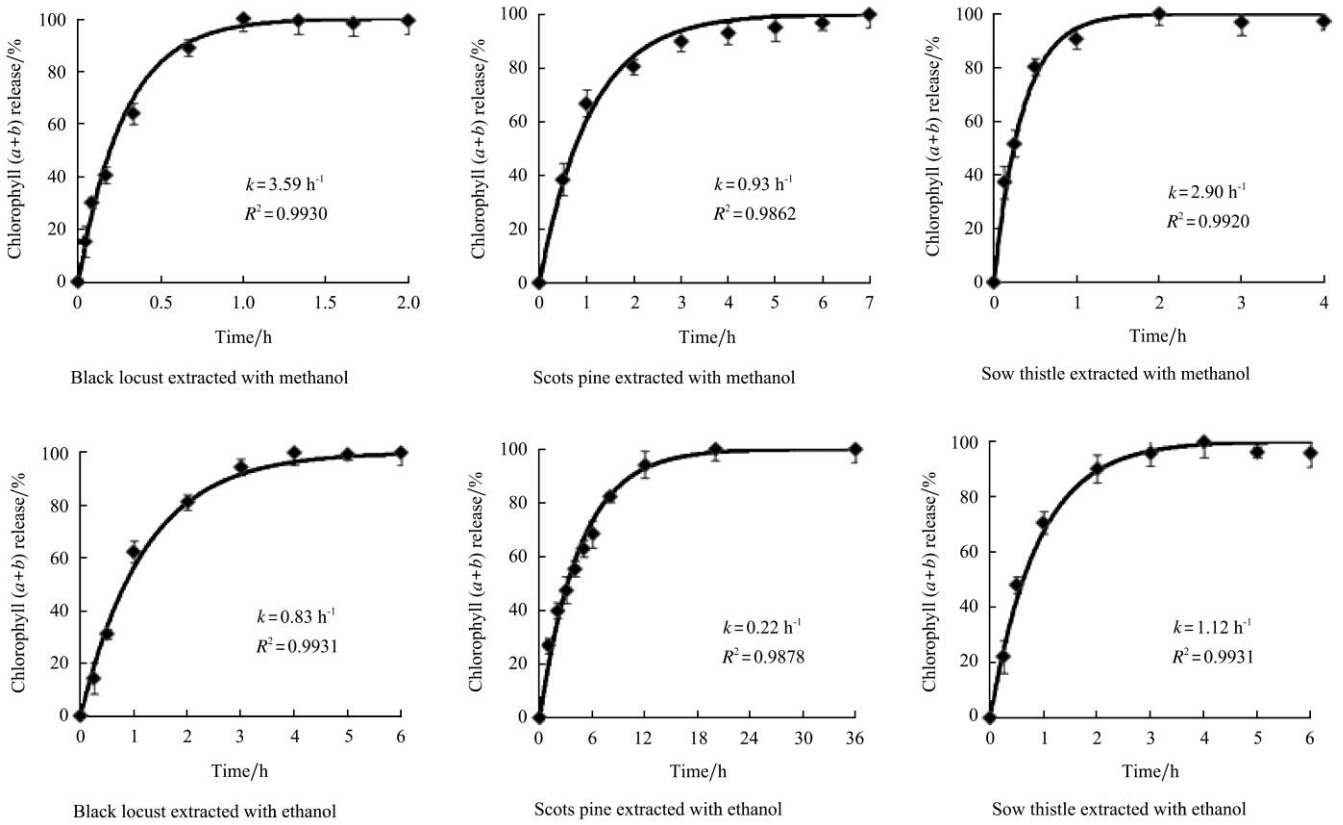


Figure 4 Kinetic model and kinetic constant (k) of chlorophyll release from black locust leaves, Scots pine needles and sow thistle leaves extracted with methanol and ethanol. All materials are from Batch 3.

Results from conducted experiments with leaves and needles showed that chlorophyll extraction rate for pine needles, during methanol treatment, was higher (k = 0.93 h^{-1}) than extraction rate for black locust leaves treated with ethanol (k = 0.83 h^{-1}). It could suggest that solvent type is more important factor than anatomy structure during extraction process. Figure 6 shows that the amount of released chlorophyll (a + b) in extracts from tested materials was almost three times higher for black locust leaves (159 μg) than for field sow thistle leaves (67 μg) and pine needles (54 μg). It means that although extraction chlorophyll rate for field sow thistle leaves treated with ethanol and pine needles extracted with methanol was higher than for black locust leaves treated with ethanol, the latter one is more favourable due to unquestionably higher amount of released chlorophyll.

Chlorophyll extraction from *Chlorella* (Figure 5) with the use of methanol, resulted in a kinetic constant (k = 2.8 h^{-1}) obtained at a level comparable with leaves. Contrary to leaves, chlorophyll extraction process of *Chlorella* culture with ethanol was characterized by a surprisingly low rate (k = 0.28 h^{-1}), as for needles. Literature data referring to the use of methanol and ethanol for chlorophyll extraction are rather contradictory. Methanol was reported to be more efficient than ethanol in terms of chlorophyll release from *Nannochloropsis gaditana* after 24-h extraction[23]. As opposite to it, ethanol showed greater than methanol efficiency of chlorophyll extraction from biological soil crust that contained algae[24]. Finally, both methanol and ethanol were recommended for chlorophyll extraction from green microalgae, with a complete extraction obtained during 24 hours[25]. What is more, in publications mentioned above, different pretreatment methods (grinding, sonication) and temperature values of extraction process (room temperature or boiling solvent) were applied, rendering our comparison even more difficult.

Cultures of microalgae are worldwide regarded as a promising source of valuable chemical compounds[26]. In this study we wanted to show that microalgae can become a good alternative for leaves and needles as a source of chlorophyll. Although, the amount of released

chlorophyll (*a* + *b*) in extracts from fresh *Chlorella* cells was only slightly higher (58 μg) than from needles (Figure 6), only 2.5 mL of culture was used before centrifugation. In case of using bigger culture volume, the amount of released chlorophyll during extraction process would be proportionally higher according to the amount of *Chlorella* culture used. Moreover, cell chlorophyll content higher than in leaves and high extraction rate with methanol, can make microalgae a good replacement for terrestrial plants. However, it must be also taken into consideration that production of chlorophyll from microalgae requires the use of high amount of biomass. High cell densities can be achieved in open ponds or closed – up photobioreactors[27], which implementation and operation can make pigment production from microalgae more difficult and expensive than from leaves or needles.

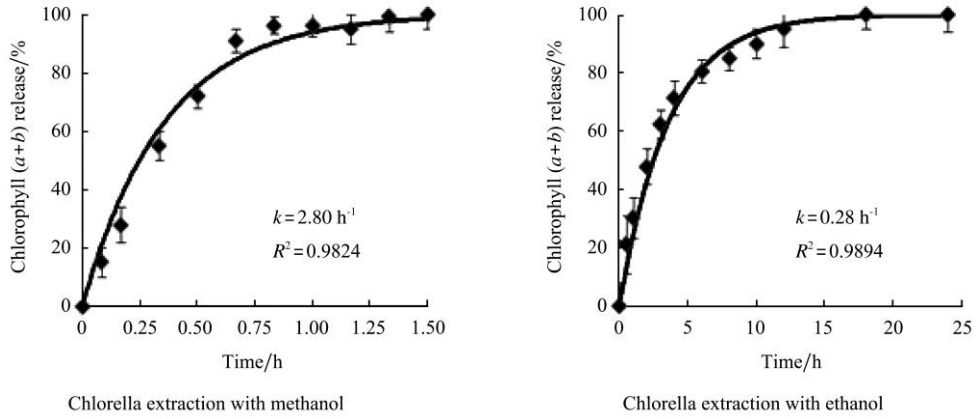


Figure 5 Kinetic model and kinetic constant (*k*) of chlorophyll release from a 32-day *Chlorella* culture extracted with methanol and ethanol

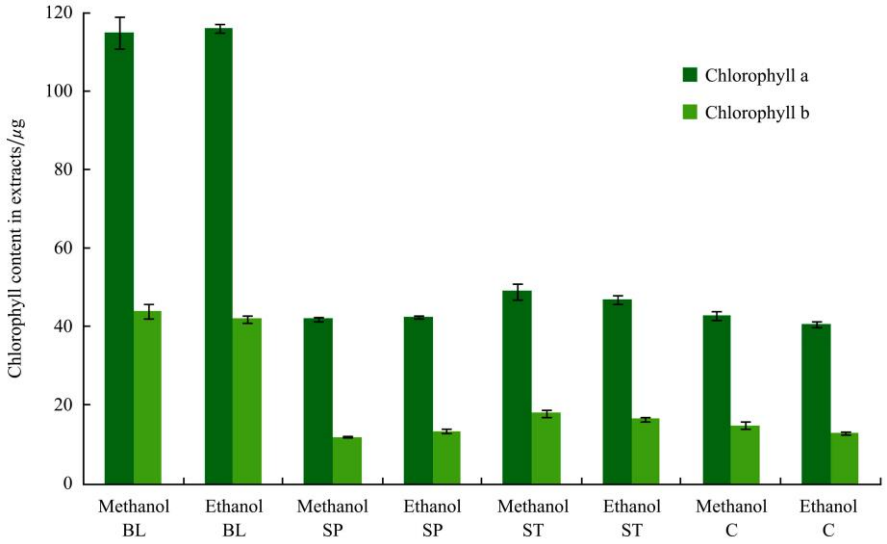


Figure 6 Chlorophyll content in extracts from plant materials treated with methanol and ethanol. (BL) Black locust leaves 250 mg of fresh material (Batch 3), (SP) Scots pine needles 250 mg of fresh material (Batch 3), (ST) Sow thistle leaves 250 mg of fresh material (Batch 3), (C) *Chlorella* sp. 2.5 mL of 32-day culture.

4 Conclusions

In this research, different plant materials were investigated in terms of possibility of chlorophyll production. The highest chlorophyll content was determined in *Chlorella* culture and in black locust leaves and the smallest in Scots pine needles. Methanol was found to outstrip ethanol and leaves and microalgae overwhelmed needles in terms of efficiency for chlorophyll extraction. Black locust leaves and *Chlorella* cells were proved to be the most attractive materials for chlorophyll production. Microalgae seem

to be the most promising alternative source for chlorophyll, as microalgae cultivation additionally helps to minimize the global warming impact due to biological CO_2 mitigation.

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